THE INTERACTION OF 1-ANILINO-8-NAPHTHALENE SULPHONATE WITH YEAST ALCOHOL DEHYDROGENASE

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1. Introduction

1-Anilino-8-naphthalene sulphonate (ANS) has been introduced as a fluorescent probe for hydrophobic areas on proteins [1, 2] and as a group for reporting changes in protein structure on the binding of allosteric ligands [3]. The binding of ANS by horse liver alcohol dehydrogenase has been reported [4] and the ANS binding site proves to be identical with the coenzyme binding site. The interaction of ANS with yeast alcohol dehydrogenase has only been reported briefly [5] and it thus seemed of interest to study this process in more detail. We find that there are probably two ANS binding sites on the enzyme which are not identical with coenzyme binding sites. It further appears that a structural change in the protein takes place on the binding of coenzyme which is reported by the fluorescent probe. ANS is found to be a weak inhibitor of enzymic activity.

2. Materials and methods

Yeast alcohol dehydrogenase was prepared from air dried baker's yeast (British Fermentation Products Ltd.) as previously described [6]. ANS was purified as the magnesium salt in the manner described by Thompson and Yielding [7]. NAD^+ and NADH (fluorimetric grade) were purchased from the Boehringer Corporation, London Ltd. Fluorescence titrations were carried out at 25° in phosphate buffer, pH 7.0, $I = 0.1$, using a Farrand Mk I spectrofluorimeter. Kinetic measurements were performed fluorimetrically at 25° in phosphate buffer pH 7.0, $I = 0.1$. Progress curves were linear for at least 30 sec. Initial rates measured in the presence of 1.03×10^{-4} M ANS were increased by a factor of 1.9 to correct for the apparently lowered fluorescence intensity of NADH in the presence of the dye.

3. Results

There is a very large increase in intensity of the fluorescence emission spectrum of ANS in the presence of yeast alcohol dehydrogenase. Accompanying this large enhancement, which results from the combination of ANS with enzyme, there is a shift in the maximum of the uncorrected emission spectrum from 520 nm to 485 nm.

Fig. 1 shows the results of fluorescence titration of 3, 6 and 30 μM ANS with enzyme. Extrapolation of the reciprocal plots yields estimates of the fluorescence of bound ANS. Within the limits of experimental error estimated values were independent of the ANS concentration. Under the conditions used here the fluorescence of ANS is enhanced some 400-fold on combination with the enzyme. The titration of 3 μM enzyme with ANS is shown in fig. 2 in the form of a Scatchard plot [8]. The data given indicate a maximum value of $\bar{\nu} = 2.1$ with a dissociation constant for the ANS binding sites of 1.3×10^{-4} M. In a total of five titration experiments we have estimated maximum values of $\bar{\nu}$ in the range 2.0 ± 0.5 and values of the dissociation constant in the range $1.2 \pm 0.1 \times 10^{-4}$ M.

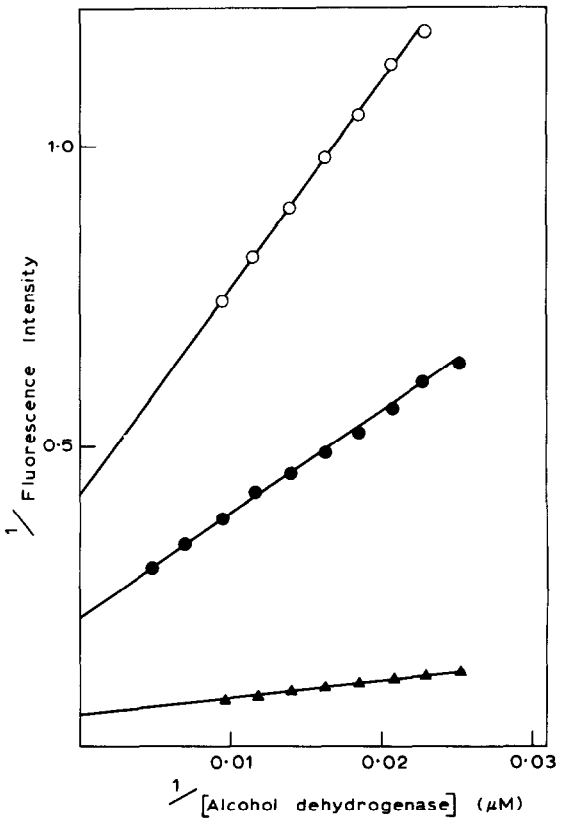

Fig. 1. Fluorescence titration of ANS with alcohol dehydrogenase at 25° and pH 7.0. ANS concentration (μM): o——o 3.0; •——• 6.0; ▲——▲ 30. Excitation at 410 nm and emission observed at 485 nm with 20 nm slits and a Corning CS 3-72 filter included between cuvette and analysing monochromator.

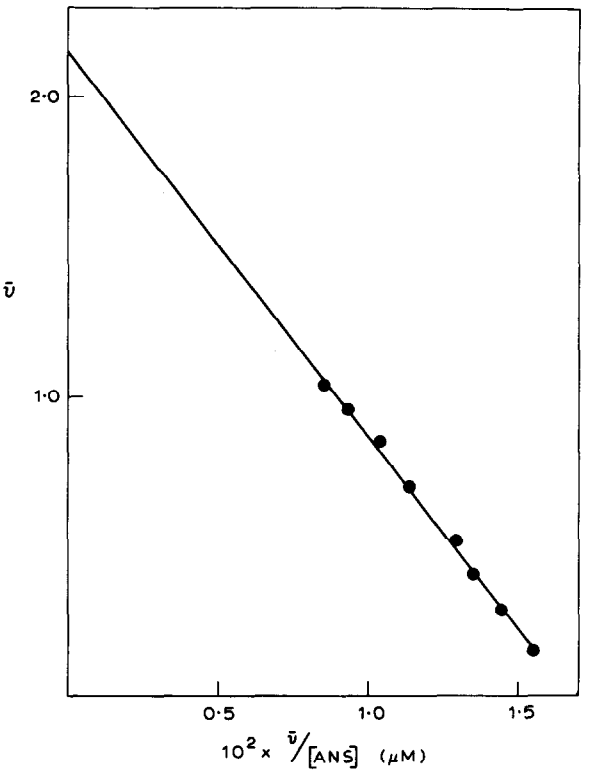

Fig. 2. Scatchard plot for the binding of ANS to enzyme at 25° and pH 7.0. Alcohol dehydrogenase (3 μM) was titrated with increasing amounts of ANS (0–120 μM). Excitation at 410 nm and emission observed at 485 nm with 20 nm slits and a Corning CS 3-72 filter included cuvette and analysing monochromator.

Fig. 3 shows the effect of NAD^+ or NADH on the fluorescence enhancement observed on the mixing of ANS and enzyme. The wavelength settings used here were chosen to minimise any contribution from NADH. Slight effects due to the intrinsic fluorescence of NADH have been corrected for. Evidently new low levels of fluorescence intensity are reached at very high concentrations of coenzyme. Repeat experiments consistently indicate that NADH produces a slightly larger effect than NAD^+.

Initial rate measurements of the enzyme catalysed oxidation of ethanol (0.54 M) with varying NAD^+ concentration (2.15×10^{-5} M–6.6×10^{-4} M) in the presence and absence of ANS (1.03×10^{-4} M) show that ANS exhibits the characteristics of a competitive inhibitor towards NAD^+. From duplicate measurements a K_i value in the range $2.5 \pm 0.3 \times 10^{-4}$ M is indicated. No inhibition of initial rate by ANS has been observed in mixtures containing high NAD^+ concentrations (1.4×10^{-3} M) with varying concentrations of ethanol (8×10^{-3} M–5.4×10^{-1} M). It has not been possible to examine the kinetics of ANS inhibition in detail. Working at higher ANS concentrations would involve the introduction of inordinately large factors, to correct measured rates for the apparent quenching of NADH fluorescence in the presence of the dye.

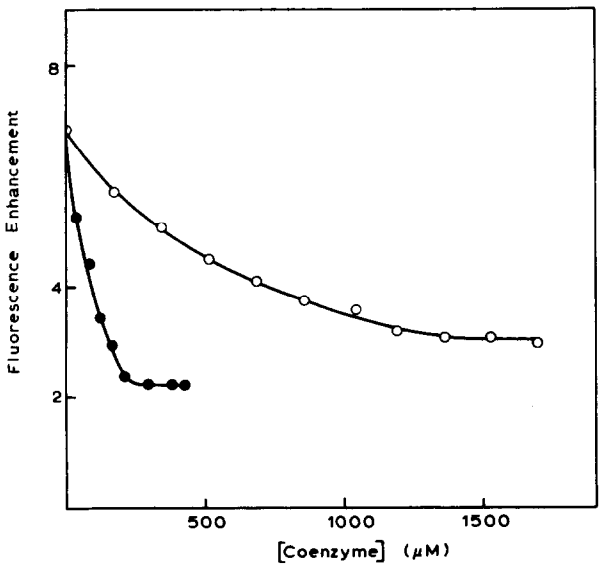

Fig. 3. Effect of NADH (●——●) and NAD^+ (○——○) on the fluorescence enhancement observed on mixing 44 μM alcohol dehydrogenase with 50 μM ANS at 25° and pH 7.0. Excitation at 410 nm and emission observed at 550 nm with 20 nm slits and a Corning CS 3-72 filter between cuvette and analysing monochromator.

4. Discussion

It is clear that ANS combines with yeast alcohol dehydrogenase to yield a complex of high relative fluorescence. The blue shift in the emission spectrum maximum and the large enhancement in the fluorescence intensity indicate that the binding sites on the protein are hydrophobic in nature [1, 2]. The polarity of these sites has already been estimated [5].

Results presented here are consistent with there being two identical ANS binding sites on the enzyme characterised by a dissociation constant of about 1.2×10^{-4} M. In view of the fact that the enzyme is known to be formed from four identical subunits [9], it seems that the ANS binding sites must be situated in a niche between subunits. Such a situation might be expected to produce a fairly hydrophobic environment.

The stoicheiometry of ANS binding precludes a direct competition between ANS and coenzymes for a common binding site. This conclusion is further supported by the results shown in fig. 4. It is clear that coenzyme concentrations of six and forty times greater than the estimated dissociation constants for these coenzymes [6, 10] do not displace all ANS from the enzyme. The situation found here is quite different from that observed in the interaction of ANS with horse liver alcohol dehydrogenase [4]. With that enzyme the single ANS binding site per subunit, characterised by a dissociation constant of 8×10^{-3} M was found to be identical with the coenzyme binding site. Evidently these binding sites are much weaker than those shown here for the yeast enzyme. Possibly the coenzyme binding sites of yeast alcohol dehydrogenase would also show a very weak interaction with ANS at much higher concentrations than we have been able to use here. However, it should be borne in mind that the specificity differences of the two alcohol dehydrogenases, towards alcohols of different chain length [11], may indicate a more extensive or more accessible hydrophobic area within the active site of the liver enzyme.

The decrease in fluorescence of mixtures containing ANS and enzyme, which results on the addition of coenzyme, presumably mirrors some conformational change in the protein following the binding of coenzyme. A change in the conformation of the enzyme on the binding of NAD^+ has already been deduced from the results of deuterium exchange studies [12]. It is of interest to note that the relative positions and general shape of the titration curves of fig. 4 are consistent with the estimated dissociation constants of NADH (10^{-5} M) and NAD^+ (2.6×10^{-4} M) from the enzyme [6, 10].

The results of kinetic measurements may be reconciled with the other results on the basis of scheme (1)

$$\begin{array}{ccc} E + NAD^+ & \overset{K_m}{\rightleftharpoons} & E.NAD^+ \xrightarrow{k_3} E + \text{products} \\ +ANS \uparrow\downarrow K_{EI} & & +ANS \uparrow\downarrow K_{EIS} \\ E.ANS + NAD^+ & \rightleftharpoons & E.ANS.NAD^+ \xrightarrow{k_3} E + \text{products} \end{array}$$

Scheme 1

where the rate constant k_3 is presumed to characterise all subsequent steps in the mechanism which are un-

changed in the presence of ANS. On the assumption that all the enzyme complexes shown are in rapid equilibrium the following reciprocal initial rate equation is derived:

$$\frac{e}{v} = \frac{1}{k_3} + \frac{K_m}{k_3} \frac{(1 + I/K_{EI})}{(1 + I/K_{EIS})} \times \frac{1}{[NAD^+]}$$

where K_{EI} and K_{EIS} are the dissociation constants for ANS from E.ANS and E.ANS.NAD^+ respectively. At a fixed inhibitor concentration the equation predicts apparently simple competitive behaviour towards coenzyme. The fact that the experimentally determined K_i (2.5×10^{-4} M) is significantly larger than the directly measured dissociation constant of ANS from the enzyme (1.2×10^{-4} M) confirms the non-identity of ANS and NAD^+ binding sites and indicates that $K_{EIS} = 3.2 \times 10^{-4}$ M. Such a value suggests that the drop in fluorescence of enzyme-ANS mixtures on the addition of NAD^+ is largely due to increased dissociation of ANS from the enzyme.ANS.NAD^+ complex, accompanied possibly by a very small decrease in the intrinsic fluorescence of bound ANS. Presumably the same applies in the case of the enzyme.ANS.NADH complex.

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